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Abstract	Pine needle diseases, such as red band and brown spot needle blight, are serious pine diseases that threatens forests in many countries. Several outbreaks have been reported resulting in loss of productivity and mortality in both exotic and native plantations of *Pinus* spp. Symptomatology of these two diseases is quite similar and characterized by the appearance of yellowish areas/bands on hosts' leaves that subsequently lead to the appearance of more extensive lesions and/or necrotic areas. In an attempt to understand the main causes of needle blight-like disease symptoms a study was carried in two pine stands that were apparently affected by red band and brown spot needle blights. Needles showing spots and/or bands with fruiting bodies were sampled. From 25 pine trees samples, 82 fungal isolates were successfully retrieved. The most common fungal genera were *Pestalotiopsis* (42.68%, n = 35), *Rhizosphaera* (28.04%, n = 23) and *Cladosporium* (9.75%, n = 8). Seven isolates could not be assigned to a species through molecular identification by ITS sequence analysis, potentially representing novel taxa. Based on multilocus phylogenetic analyses, using ITS, *tub2* and *tef1-α* sequences, and morphological data, we propose three novel fungal species: *Didymocyrtis pini* sp. nov., *Pestalotiopsis iberica* sp. nov. and *Rhizosphaera pinicola* sp. nov. These species are potential active players in the symptomatology initially associated to red band and brown spot needle blight diseases. Although the pathogenicity of these fungi needs to be confirmed, this study suggests a high complexity underlying fungal species associated with these diseases which may impact disease epidemiology and management.

Keywords (separated by '-')	Emergent diseases - Forest pathogens - Fungal diversity - Pine needle blight diseases - Needle blight - Needle cast
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Three novel species of fungi associated with pine species showing needle blight-like disease symptoms

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Abstract Pine needle diseases, such as red band and brown spot needle blight, are serious pine diseases that threatens forests in many countries. Several outbreaks have been reported resulting in loss of productivity and mortality in both exotic and native plantations of *Pinus* spp. Symptomatology of these two diseases is quite similar and characterized by the appearance of yellowish areas/bands on hosts' leaves that subsequently lead to the appearance of more extensive lesions and/or necrotic areas. In an attempt to understand the main causes of needle blight-like disease symptoms a study was carried in two pine stands that were apparently affected by red band and brown spot needle blights. Needles showing spots and/or bands with fruiting bodies were sampled. From 25 pine trees samples, 82 fungal isolates were successfully retrieved. The most common fungal genera were *Pestalotiopsis* (42.68%, n = 35), *Rhizosphaera* (28.04%, n = 23) and *Cladosporium* (9.75%, n = 8). Seven isolates could not be assigned to a species through molecular identification by ITS sequence analysis, potentially representing novel taxa. Based on multilocus phylogenetic analyses, using ITS, *tub2* and *tef1-α* sequences, and morphological data, we propose three novel fungal species: *Didymocyrtis pini* sp. nov., *Pestalotiopsis iberica* sp. nov. and *Rhizosphaera pinicola* sp. nov. These species are potential active players in the symptomatology initially associated to red band and brown spot needle blight diseases. Although the pathogenicity of these fungi needs to be confirmed, this study suggests a high complexity underlying fungal species associated with these diseases which may impact disease epidemiology and management.

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Introduction

Conifer forests, where *Pinus* species are included, occupy approximately 104 million ha of the European territory which represents a valuable asset for the green economy (Korhonen & Stahl, 2020). In the Mediterranean region, this ecosystem is one of the most explored natural resources providing us with a wide array of goods (wood and non-wood products) and services, making this ecosystem crucial for the socio-economic development (European Forest Institute (EFI), 2020).

Climate change as well as the introduction and interchange of new and/or exotic plant reproductive material (species, provenances, hybrids and/or clones) may boost the most common fungal diseases that attack pine needles (Bednářová et al., 2013). Pine needle blight diseases (PNBD) are caused by fungal species belonging to the well-known Ascomycota classes, such as *Leotiomycetes*, *Dothideomycetes* (e.g. *Mycosphaerella*, *Dothistroma* and *Lecanosticta* spp.) and *Sordariomycetes* which have been linked with several needle blight diseases such as Dothistroma Needle Blight (caused by *Dothistroma pini* and *D. septosporum*), Brown-Spot Needle Blight (caused by *Lecanosticta acicola*) or Lophodermium Needle Cast (caused by *Lophodermium seditiosum*) (Bednářová et al., 2013). Pine needle blight diseases and needle casts are commonly found in nurseries, plantations, forest and/or urban stands being characterized by the occurrence of needle discolorations and needle spots that lead to necrotic lesions consequently reducing leaf area and photosynthetic efficiency and increasing crown defoliation threatening plants' healthiness and productivity (Bednářová et al., 2013). Recently, PNBD has received special attention due to several outbreaks that have been reported in European countries such as Spain, Switzerland, Latvia, Ireland, Bulgaria and Portugal in both native and exotic plantations as well as in nurseries (Janssons et al., 2020; Mullett et al., 2018; Ortíz de Urbina et al., 2016; Schneider et al., 2019). Currently, *L. acicola* has been listed as A2 pest being classified as quarantine species by EPPO (European and Mediterranean Plant Protection Organization (EPPO), 2020). The main bottleneck of these diseases identification is the similarity of symptomatology, leading to misidentifications in field conditions (Schneider et al., 2019) that may compromise the correct diagnosis and subsequent appropriated control. In the field, plants can frequently be infected by more than one pathogenic species at the same time. Furthermore, there are strong evidences highlighting the complexity behind plant disease including the synergisms between different pathogens that may contribute to similar symptoms (Lamichhane & Venturi, 2015). Overall, there is a need to clarify the agents involved in PNBD.

Considering this, and within the scope of our recent studies on pine's fungal pathogens, this study aimed to assess the diversity of fungi occurring, and identify potential new fungal species, in several pine species from specific areas where PNBD-like symptoms were known to occur. Furthermore, the study of the fungal communities in PNBD is important to disclose key fungal species and occurring synergies that play an important role in disease's dynamics.

Materials and methods

Sampling procedure

Sampling sites were located in Puentenansa, Cantabria, Spain (Fig. 1A and Table S1) near to recently described outbreak sites of *D. septosporum* and *L. acicola* (Mesanza et al., 2021). Local climatic conditions are characterized by having average annual warm temperatures (10–14 ◦C), high precipitation levels (700–2400 mm per year) and elevation ranging from 0 to 1000 m above the sea level (Blank et al., 2019).

Trees were visually inspected for symptoms (spots or bands with fruiting bodies) and symptomatic needles were collected (Fig. 1B). Samples were stored at 4 ºC until further processing. A total of 25 pine trees were sampled including *Pinus radiata* (nine trees, representing 36% of total samples), *P. sylvestris* (six trees, 24%), *P. nigra* (four trees, 16%), *P. pinaster* (four trees, 16%) and *P. uncinata* (two trees, 8%) (Table S1).

Fungal isolation and culture growth

Fungal cultures were obtained following the spore streaking method outlined by Mullett and Barnes (2012). Briefly, fruiting bodies were excised from each symptomatic needle under a dissecting zoom stereomicroscope (SMZ1500, Nikon Instruments Europe B.V., Amstelveen, Netherlands) and crushed on a glass microscope slide in a drop of sterile distilled water and the presence of conidia confirmed under a light microscope (Nikon Eclipse 80i, Nikon

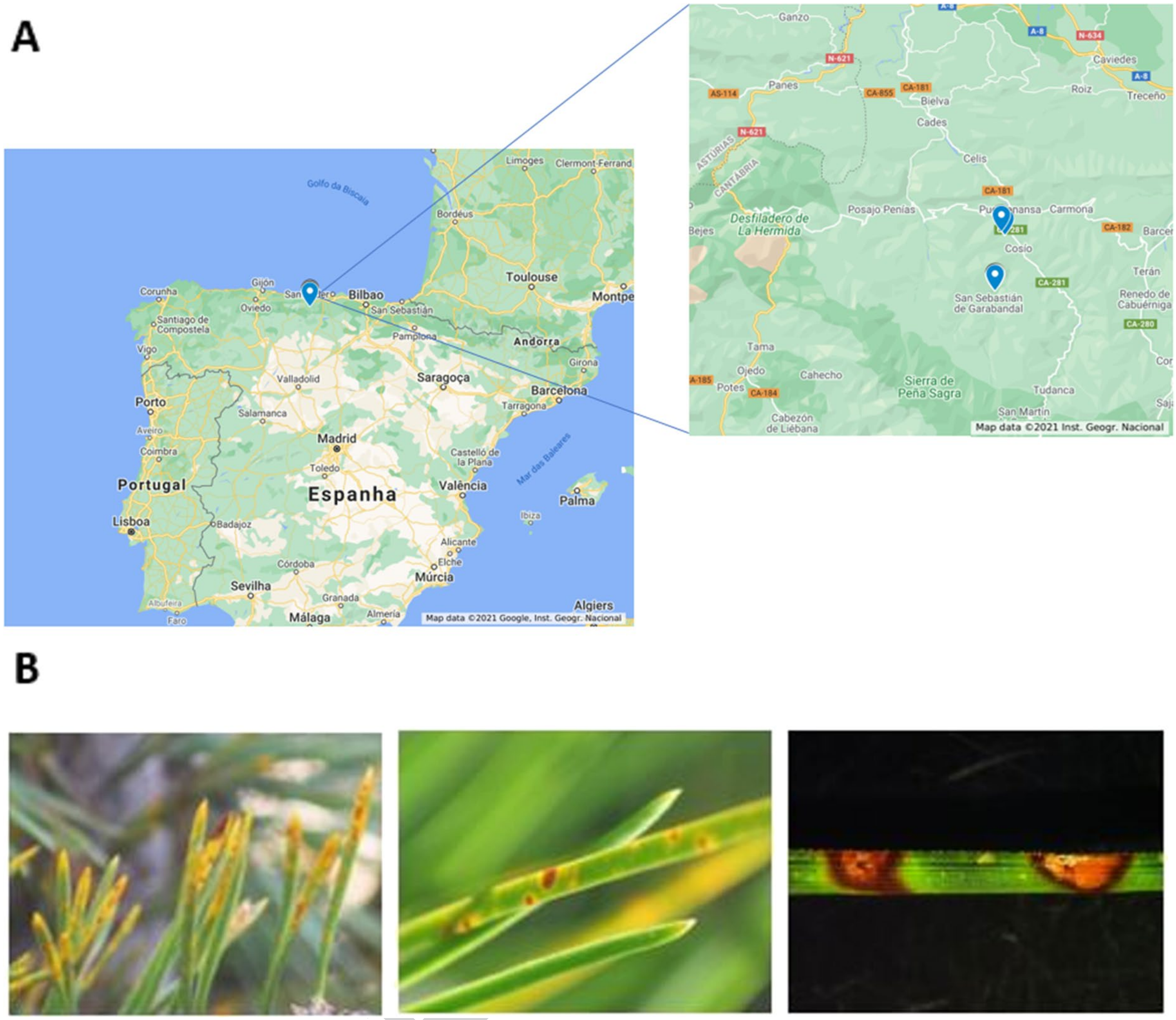

Fig. 1 **A**. Sampling sites used in this study. **B**. Representative images from collected needles showing Pine Needle Blight Disease (PNBD) symptoms

Instruments Europe B.V., Netherlands) coupled to a high-resolution digital microscope camera (DS-Ri1, Nikon Instruments Europe B.V.) and its controller (DS-U3, Nikon Instruments Europe B.V.). The NIS-Elements Documentation imaging software (v. 64bit 3.22.15, Nikon Instruments Europe B.V.) was used for image acquisition. Using an inoculation loop, spore solution was spread on a Petri dish containing *Dothistroma* medium (DM) supplemented with streptomycin to prevent bacterial growth (Mullett & Barnes, 2012). Growing cultures were daily observed under the microscope and once growing hyphae were visible, these were isolated in DM medium obtaining fungal pure cultures that were posteriorly maintained on DM at room temperature (approximately 20 ºC).

Genomic DNA extraction, PCR amplification, sequencing and phylogenetic analysis

Genomic DNA was extracted from fresh mycelium of cultures growing on DM (Möller et al., 1992). Microsatellite-primed PCR (MSP-PCR) with GTG5 primer (5'-GTGGTGGTGGTGGTG-3') was used for molecular typing of all isolates (Alves et al., 2007). Analysis of the genetic fingerprinting patterns was performed with GelCompar II software

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(Applied Maths). Pearson correlation coefficient was calculated, and cluster analysis was performed using unweighted pair group method with arithmetic mean (UPGMA) algorithm. Resulting dendrograms were analysed in order to obtain groups of isolates with at least 80% similarity. This cut-off was determined so that patterns that were known to be equal would be considered to be in the same cluster. Representative isolates of each group were randomly selected and subjected to PCR amplification of the ITS region of the rDNA using primers ITS1 and ITS4, following the cycling conditions previously described (Alves et al., 2004). Amplified PCR fragments were purified with NZYGelpure kit (NZYTech) before sequencing at GATC Biotech (Cologne, Germany). The nucleotide sequences were analysed with FinchTV version 1.4.0 (Geospiza). A BLASTn search against the NCBI nucleotide collection (nr/nt) database was carried out to determine the closest matching sequences. Information from representative isolates was used to associate taxonomic affiliation with all isolates from the collection. Seven isolates (CAA 1002, CAA 1003, CAA 1004, CAA 1005, CAA 1006, CAA 1007 and CAA 1008) could not be affiliated to any of the currently known species. Therefore, taking into account that ITS results only provide information at genus level, additional molecular markers were used for amplification and sequencing of the unknown isolates according to the genus identified. For CAA 1002/CAA 1003 and CAA 1007/CAA 1008 and: beta-tubulin (*tub2*) with the primer set T1/Bt2b and Bt2a/Bt2b, respectively. For CAA 1004, CAA 1005 and CAA 1006: *tub2* with T1/Bt2b and translation elongation factor 1 alpha (*tef1-α*) with the EF1-688F/EF1-1251R primers set. Amplification conditions were followed accordingly to Lopes et al. (2017) and Hunter et al. (2006). The amplicons were purified, sequenced and used as query in BLASTn analyses as described above. The closest sequences were then used in sequence alignment to determine the taxonomic affiliation of the isolates in a single and multi-locus sequence analysis approach. Sequences were aligned with ClustalX version 2.1 (Thompson et al., 1997) using the parameters described in Gonçalves et al. (2020). All alignments were checked and edited with BioEdit Alignment Editor version 7.2.5 (Hall, 1999). Phylogenetic analyses were done with MEGAX version 10.0.4 (Kumar et al., 2018). The best substitution model to build the maximum-likelihood (ML) tree was performed on a neighbour-joining starting tree automatically generated by the software. Nearest-neighbour-interchange was used as the heuristic method for tree inference with 1000 bootstrap replicates. Maximum-parsimony analyses were performed with PAUP version 4.0b10 (Swofford, 1993) according to Gonçalves et al. (2020). Bayesian inference was performed using Mr. Bayes version 3.0b4 (Ronquist & Huelsenbeck, 2003) according to Gonçalves et al. (2020). The sequences generated in this study were deposited in GenBank and taxonomic novelties in MycoBank (Table 1). Alignment and trees were deposited in TreeBase (TB2:S27971).

Morphology and growth studies

Temperature growth studies were performed for the new species described on potato dextrose agar (PDA), malt extract agar (MEA) and water agar (WA) culture media. Three replicate plates per isolate were incubated at 5, 10, 15, 20, 25, 30 and 35 ºC in the dark. Colony diameter was measured daily for 14 days. Colony characters and morphological descriptions were registered after 14 days of growth at 25 ºC based on sporulating cultures. The colour charts of Werner's were used to characterize the culture colours (obverse and reverse) (Syme, 2018). Observations of macro-morphological characters were registered under a stereomicroscope (SMZ 1500, Nikon Instruments Europe B.V., Amstelveen, Netherlands). Fungal structures (mainly conidiophores, conidiogenic cells, conidiomata and conidia) were mounted in 100% lactic acid and observed under a light microscope with differential interference contrast (Nikon Eclipse 80i, Nikon Instruments Europe B.V., Netherlands). Photographs were captured through a high-resolution digital camera (DS-Ri1, Nikon Instruments Europe B.V.) and its controller (DS-U3, Nikon Instruments Europe B.V.).

Results

Diversity of fungal isolates

A total of 82 fungal isolates were retrieved from fruiting bodies of the above-mentioned *Pinus* species symptomatic needles. The majority belonged to the genera *Pestalotiopsis* (42.68%, n = 35), *Rhizosphaera*

Table 1 List of isolates used in this study

Species	Strain	Host/substrate	Country	Accession Numbers		
				ITS	*tub2*	*tef1-α*
Didymocyrtis banksiae	CSN1065	*Olea europea*	South Africa	MT813919		
	CSN1049	*Olea europea*	South Africa	MT813909		
	CBS 142523*	*Banksia sessilis* var. *cygnorum*	Australia	KY979757	KY979923	
Didymocyrtis brachylaenae	CPC 32651*	*Brachylaena discolor*	South Africa	MH327821	MH327896	
Didymocyrtis cladonicola	UTHSC DI16-330	respiratory tract	USA	LT796886	LT796966	
	UTHSC DI16-313	respiratory tract	USA	LT796877	LT796957	
	CBS 131733	*Cladonia rangiformis*	France	KP170646	KP170696	
	CBS 131732	*Cladonia symphycarpa*	France	KP170645	KP170695	
	CBS 131731	*Ramalina pollinaria*	France	KP170644	KP170694	
	CBS 128027	*Parmelina tiliacea*	Spain	KP170643	KP170693	
	CBS 128026	*Cladonia* sp.	Spain	KP170642	KP170692	
	CBS 128025	*Squamarina cartilaginea*	Belgium	KP170641	KP170691	
	CBS 128023	*Squamarina cartilaginea*	Belgium	KP170640	KP170690	
Didymocyrtis consimilis	CBS 129140	*Caloplaca cerina*	Canada	MH865190		
	CBS 129338	*Caloplaca cerina*	Canada	MH865230		
Didymocyrtis epiphyscia	Freebury 1411	*Physcia aipolia*	Canada	KT383824		
Didymocyrtis foliaceiphila	CBS 129141	*Cladonia squamosa*	Belgium	MH865191	KP170698	
	CBS 131730	*Parmelia sulcata*	Belgium	KP170650	KP170700	
	CBS 131729	*Cladonia* spp.	Belgium	KP170649	KP170699	
Didymocyrtis melanelixiae	Harris 57476	*Punctelia rudecta*	USA	KT383831		
	Harris 57465	*Cetrelia olivetorum*	USA	KT383830		
	Harris 57475	*Punctelia rudecta*	USA	KT383828		
Didymocyrtis pini	**CAA 1002***	***Pinus radiata***	**Spain**	**MW732246**	**MW759031**	
	CAA 1003	***Pinus radiata***	**Spain**	**MW732247**	**MW759030**	
Didymocyrtis pseudeverniae	Diederich 17338	*Pseudevernia furfuracea*	Switzerland	KT383834		
	Diederich 17327b	*Pseudevernia furfuracea*	Switzerland	KT383833		
	Diederich 17327a	*Pseudevernia furfuracea*	Switzerland	KT383832		
Didymocyrtis ramalinae	Paul 10i13	*Ramalina fastigiata*	Scotland	KT383839		
	Ertz 16399	*Ramalina* spp.	France	KT383838		
	Paul 27i13	*Ramalina fastigiata*	Scotland	KT383836		
Didymocyrtis slaptoniensis	MoraB	*Xanthoria parietina*	France	KT383842		
	MoraA	*Xanthoria parietina*	France	KT383841		
	Gardiennet 12009	*Xanthoria parietina*	France	KT383840		
Didymocyrtis trassii	AB298	*Cetraria aculeata*	Ukraine	MG519614		
	AB297	*Cetraria aculeata*	Ukraine	MG519613		
	VO271	*Cetraria aculeata*	Ukraine	MG519611		
Didymocyrtis xanthomendozae	CBS 129666*	on fallen *Salix*	Canada	KP170651	KP170701	
Pestalotiopsis iberica	**CAA 1004***	***Pinus radiata***	**Spain**	**MW732248**	**MW759035**	**MW759038**
	CAA 1005	***Pinus sylvestris***	**Spain**	**MW732250**	**MW759034**	**MW759037**
	CAA 1006	***Pinus radiata***	**Spain**	**MW732249**	**MW759036**	**MW759039**
Pestalotiopsis clavata	MFLUCC 12-0268*	*Buxux* sp.	China	JX398990	JX399025	JX399056

Table 1 (continued)

Species	Strain	Host/substrate	Country	Accession Numbers		
				ITS	*tub2*	*tef1-α*
Pestalotiopsis lushanensis	LC8182	*Camelia* sp.	China	KY464136	KY464156	KY464146
	LC4344*	*Camelia* sp.	China	KX895005	KX895337	KX895223
Pestalotiopsis pini	CBS 146840*	*Pinus pinea*	Portugal	MT374680	MT374705	MT374693
	CBS 146841	*Pinus pinea*	Portugal	MT374681	MT374706	MT374694
Pestalotiopsis rhododendri	CBS 144024	*Pinus* sp.	Zimbabwe	MH554109	MH554782	MH554543
	IFRDCC 2399*	*Rhododendron sinogrande*	China	KC537804	KC537818	KC537811
Rhizosphaera kalkhoffii	CBS 280.38	*Pinus densiflora*	Korea	EU700375	EU747273	
	CBS 114656	*Pinus densiflora*	Korea	EU700376	EU747274	
Rhizosphaera kobayashii	ATCC 46389	*Pinus pumila*	Japan	AF462432		
Rhizosphaera macrospora	ATCC 4636	*Abies alba*	France	AF462431		
	CBS 467.82	*Pinus densiflora*	Korea	EU700368	EU747280	
	CBS 208.79*	*Abies alba*	France	MH861202		
Rhizosphaera oudemansii	ATCC 46390	*Abies alba*	France	AF462430		
	CBS 226.83	*Pinus densiflora*	Korea	EU700366	EU747278	
	CBS 427.82	*Pinus densiflora*	Korea	EU700367	EU747279	
Rhizosphaera pini	CBS 189.26	-	Netherlands	MH854884		
	DAOMC251499	House dust	Canada	KY659500	KY659498	
	CBS 206.79	*Pinus densiflora*	Korea	EU700370	EU747282	
Rhizosphaera pinicola	**CAA 1007***	***Pinus nigra***	**Spain**	**MW732245**	**MW759033**	
	CAA 1008	***Pinus radiata***	**Spain**	**MW732244**	**MW759032**	
Rhizosphaera pseudotsugae	CBS 101222*	*Pinus densiflora*	Korea	EU700369	EU747281	
Neopestalotiopsis saprophytica	MFLUCC 12-0282*	*Magnolia* sp.	China	KY606286	JX399017	JX399048

ATCC American Type Culture Collection; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CAA: Culture collection of Artur Alves, housed at Department of Biology, University of Aveiro, Aveiro, Portugal; CPC: Culture collection of Pedro Crous, housed at CBS; CSN: Collection of Chris Spies at ARC-Nietvoorbij, Stellenbosch, South Africa; DAOMC: Canadian Collection of Fungal Cultures hosted at Agriculture and Agri-Food Canada; IFRDCC: International Fungal Research and Development Culture Collection, Yunnan, China; LC: working collection of Lei Cai, housed at the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; MUM: Culture collection hosted at Center for Biological Engineering of University of Minho, Braga, Portugal; UTHSC: University of Tennessee Health Science Center. Ex-type strains are marked with an asterisk. Sequences generated in this study are shown in bold

(28.04%, n = 23) and *Cladosporium* (9.75%, n = 8). Genera such as *Botrytis* (3.65%, n = 3), *Didymocyrtis* (3.65%, n = 3), *Umbelopsis* (3.65%, n = 3), *Alternaria* (1.21%, n = 1), *Fusarium* (1.21%, n = 1), *Epicoccum* (1.21%, n = 1), *Neophysalospora* (1.21%, n = 1), *Penicillium* (1.21%, n = 1) and *Sydowia* (2.43%, n = 2) were also present within sampled needles (Fig. 2).

In this study, fungal isolation from symptomatic needles of *P. uncinata* was not successful (0%, n = 0) (Fig. 3). *Umbelopsis isabellina* (3.7%, n = 3), *Cladosporium herbarum/allicum* (2.4%, n = 2), *Cladosporium cladosporioides* (1.2%, n = 1) and *Fusarium reticulatum/culmorum/avenaceum* (1.2%, n = 1) only colonized *P. sylvestris*. Likewise, *Botrytis cinerea* (3.7%, n = 3), *Didymocyrtis* sp. (3.7%, n = 3), *Pestalotiopsis biciliata/neglecta/microspora* (3.7%, n = 3), *Sydowia polyspora* (2.4%, n = 2), *Alternaria rosae* (1.2%, n = 1), *Cladosporium cladosporioides/tenuissimum/varians* (1.2%, n = 1), *Cladosporium colombiae* (1.2%, n = 1), *Cladosporium xanthochromaticum/regulovarians/delicatulum* (1.2%, n = 1), *Epicoccum nigrum* (1.2%, n = 1) and

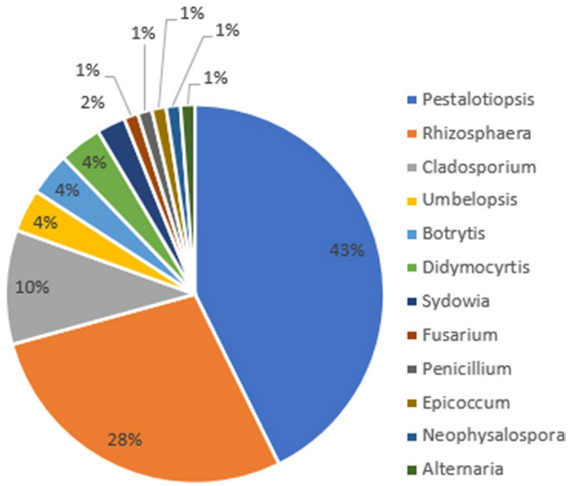

Fig. 2 Distribution of the 21 fungal genera retrieved from the sampled pine needles

Neophysalospora eucalypti (1.2%, n = 1) were exclusively isolated from *P. radiata*. Regarding *P. pinaster*, only *Cladosporium perangustum* (1.2%, n = 1), *Penicillium corylophilum* (1.2%, n = 1) and *Pestalotiopsis verruculosa* (1.2%, n = 1) fungal species were successfully isolated. In *P. nigra*, *Rhizosphaera* sp. was the only fungal species retrieved.

Molecular characterization

The isolates that could not be affiliated to any of the currently known species may represent potential novel taxa. Thus, representative isolates of *Didymocyrtis* sp. (CAA 1002 and CAA 1003), *Pestalotiopsis* sp. (CAA 1004, CAA 1005 and CAA 1006) and *Rhizosphaera* sp. (CAA 1007 and 1008) were selected to be further characterized.

The closest hits using the ITS sequence for CAA 1002 and CAA 1003 belonged to uncultured fungus clone ZMTCB201207-33 (GenBank accession no. KX516501; identities 581/581 [100%], 0 gaps), Fungal sp. mh3037.5 (GenBank accession no. GQ996135; identities 581/581 [100%], 0 gaps) and *Didymocyrtis cladoniicola* UTHSC:DI16-330 (GenBank accession no. LT796886; identities 581/581 [100%], 0 gaps). Closest hits using *tub2* sequence had highest similarities to *D. cladoniicola* UTHSC:DI16-330 (GenBank accession no. LT796966; identities 365/367 [99%], 0 gaps). Single-gene data sets with ITS sequences were aligned separately with those of all known *Didymocyrtis* species to access which species are closest to our isolates before performing a multilocus phylogenetic analysis (Table 1). To confirm the phylogenetic placement of the putative novel species within the genus *Didymocyrtis*, *tub2* was also sequenced and both single and combined phylogenetic trees were performed. Single-locus trees (ITS and *tub2*) are given in Fig. S1 and S2. The alignment of combined ITS + *tub2* contained 18 sequences (including outgroup), and there was a total of 1043 positions in the final data set. In the ML combined ITS + *tub2* phylogenetic tree (Fig. 4), isolates CAA 1002 = MUM 21.03 and CAA 1003 clustered into one distinct clade that received high bootstrap support (99–100%) and high PP values (1.00) within the genus *Didymocyrtis* and together with one isolate named as *D. cladoniicola* UTHSC:DI16-330. However, according to our phylogenetic analysis, our isolates (CAA 1002/1003) and UTHSC:DI16-330 clustered together and are clearly separated from the other well defined *D. cladoniicola* clade, which includes a series of cultures (CBS collection) that can be regarded as authentic and representative of *D. cladoniicola*. In addition, UTHSC:DI16-330 and UTHSC:DI16-313 were identified based only on D1-D2 of LSU sequences and, none of these isolates grouped with *D. cladoniicola* CBS 128,025, suggesting that probably both isolates are miss-identified (Valenzuela-Lopez et al., 2017). Therefore, based on our molecular data and on the available morphological descriptions (further discussed in the Taxonomy section) we propose a new species name that encompass our isolates.

Regarding isolates CAA 1004, CAA 1005 and CAA 1006, the closest matches for ITS sequence were *Pestalotiopsis* sp. 38 (GenBank accession no. KP900727; identities 545/545 [100%], 0 gaps) and *Pestalotiopsis* sp. ATT181 (GenBank accession no. HQ607878; identities 540/545 [99%], 0 gaps). Single phylogenetic tree with ITS sequences was aligned separately with those of all known *Pestalotiopsis* species to access which species are closest to our isolates before performing a multilocus phylogenetic analysis (Table 1). The *tub2* and *tef1-α* genes were also sequenced to confirm the phylogenetic placement in *Pestalotiopsis*. The highest similarities using the *tub2* sequence were *Pestalotiopsis lushanensis* LB32-3 (GenBank accession no. MG726541; identities 759/763 [99%], 0 gaps)

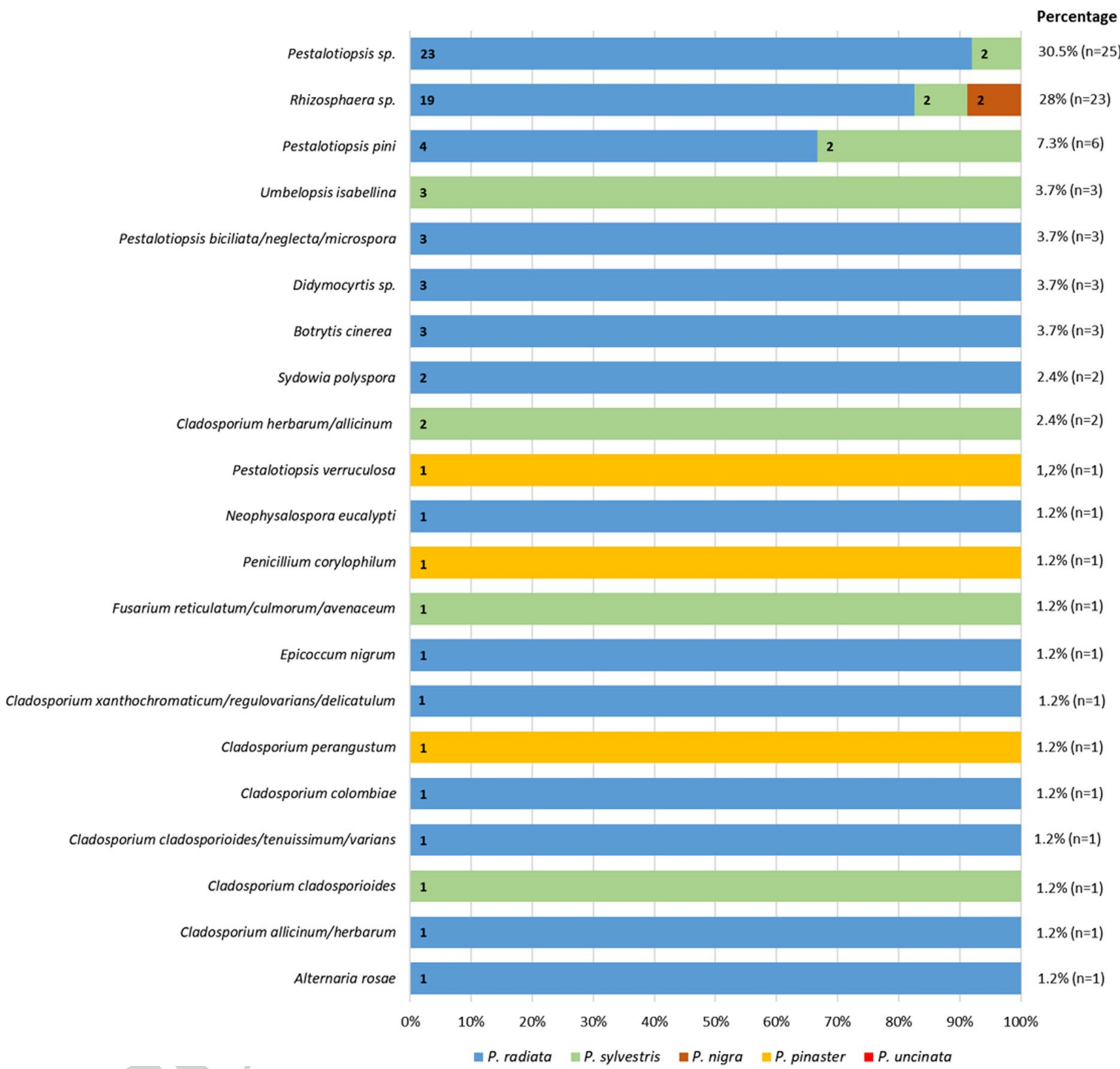

Fig. 3 Distribution of the 21 fungal species among *Pinus* spp. retrieved from the sampled pine needles

and *P. pini* MEAN 1095 (GenBank accession no. MT374707; identities 759/763 [99%], 0 gaps). Using the *tef1-α* sequence, the highest similarities were *P. lushanensis* LC4344 (GenBank accession no. KX895223; identities 542/544 [99%], 0 gaps) and *P. clavata* MFLUCC12-0269 (GenBank accession no. JX399057; identities 542/544 [99%], 0 gaps). Therefore, ITS, *tub2* and *tef1-α* sequences of CAA 1004, CAA 1005 and CAA 1006 were aligned with the closest *Pestalotiopsis* species based on ITS single phylogenetic analysis. The alignment of combined related species of ITS + *tub2* + *tef1-α* contained 11 sequences (including outgroup), and there was a total of 1455 positions in the final data set. In the ML combined ITS + *tub2* + *tef1-α* phylogenetic tree (Fig. 5), isolates CAA 1004 = MUM 21.02, CAA 1005 and CAA 1006 clustered into one distinct clade that received high bootstrap support (96–99%) and high PP values (1.00) within the genus *Pestalotiopsis* with close relationship with *P. lushanensis*.

Regarding isolates CAA 1007 and CAA 1008, the closest matches for ITS sequence belonged

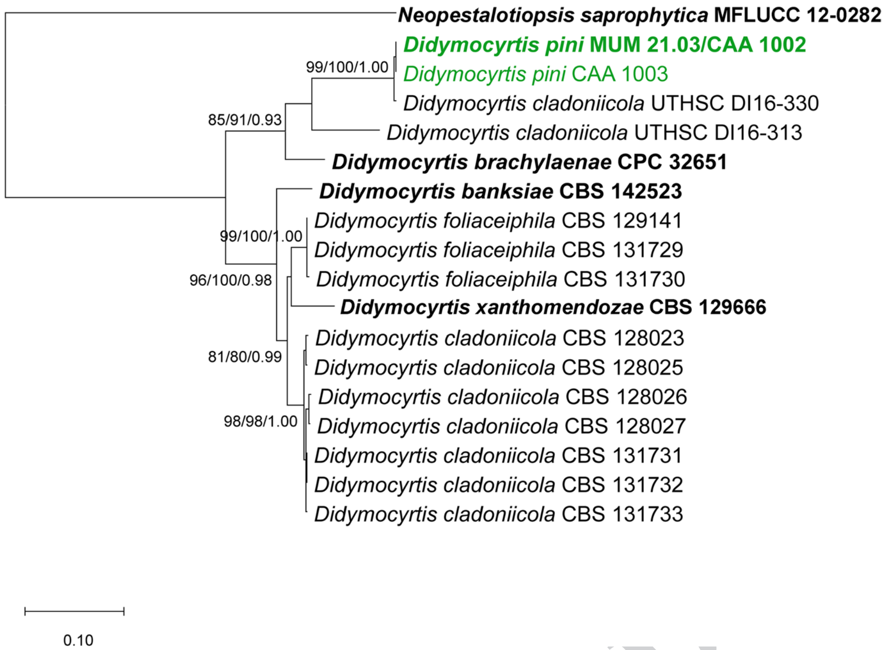

Fig. 4 Phylogenetic relationships of *Didymocyrtis* species based on combined ITS and *tub2* sequence data and inferred using the maximum-likelihood (ML) method under the Kimura two-parameter model with gamma distribution. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Neopestalotiopsis saprophytica* MFLUCC 12-0282. Bootstrap values (≥ 70%) of ML and maximum-parsimony (MP) analyses and posterior probabilities (≥ 0.90) of Bayesian inference (BI) are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the new taxon proposed from the current study is given in green

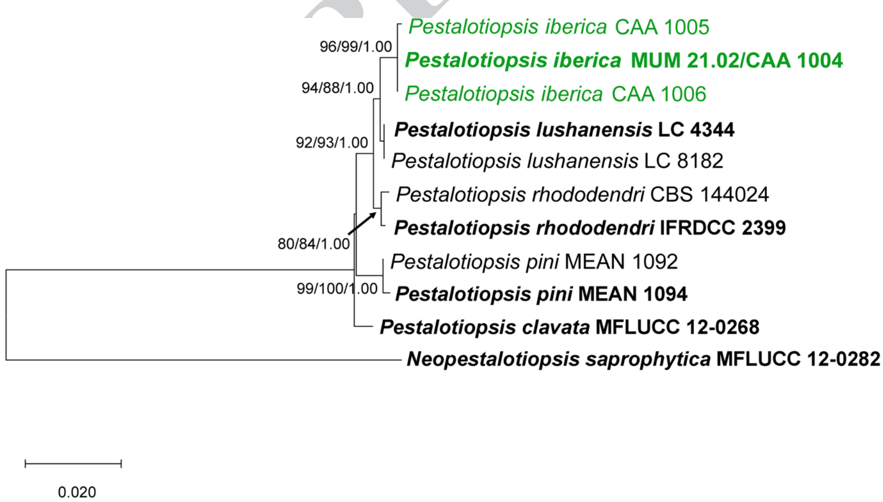

Fig. 5 Phylogenetic relationships of *Pestalotiopsis* species based on combined ITS, *tub2* and *tef1-α* sequence data and inferred using the maximum-likelihood (ML) method under the Kimura two-parameter model with uniform rates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Neopestalotiopsis saprophytica* MFLUCC 12–0282. Bootstrap values (≥ 70%) of ML and maximum-parsimony (MP) analyses and posterior probabilities (≥ 0.90) of Bayesian inference (BI) are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the new taxon proposed from the current study is given in green

to unidentified isolates, such as *Rhizosphaera* sp. ZLY-2010b isolate M77-2 (GenBank accession no. HM595558; identities 547/551 [99%], 0 gaps) and Dothideomycetes sp. 668 JMUR-2016 (GenBank accession no. KX908516; identities 541/551 [98%], 9 gaps). The *tub2* gene was also sequenced to confirm the phylogenetic placement in *Rhizosphaera*. The highest similarities using the *tub2* sequence were also unidentified *Rhizosphaera* isolates, such as *Rhizosphaera* sp. P01401 (GenBank accession no. EU747277; identities 342/360 [95%], 0 gaps) and *Rhizosphaera* sp. P02011 (GenBank accession no. EU747276; identities 341/360 [95%], 0 gaps). The closest match of an identified species was *R. macrospora* CBS 467.82 (GenBank accession no. EU747280; identities 330/361 [91%], 2 gaps). ITS and *tub2* sequences of CAA 1007 and CAA 1008 was aligned with those of all *Rhizosphaera* species to confirm the phylogenetic placement in this genus (Table 1). The alignment contained 11 sequences (including outgroup), and there was a total of 968 positions in the final data set. Combined ML phylogenetic tree is given in Fig. 6, whereas single-locus trees (ITS and *tub2*) in Fig. S3 and S4. Our isolates clustered into one distinct clade that received high bootstrap support (100%) and high PP values (1.00) with a close relationship to *R. pseudotsugae*.

Taxonomy

Didymocyrtis pini P. Monteiro & M. Gonçalves, sp. nov. (Fig. 7)

MycoBank: MB 840198

Typification: Puentenansa, Cantabria, Spain. (43°14′20.5"N 4°24′41.1"W), isolated from symptomatic *Pinus radiata* needles, 21 November 2019, P. Monteiro, deposited in the Micoteca of Universidade do Minho (holotype a dried culture sporulating, MUM-H 21.03). Ex-type living culture MUM 21.03 = CAA 1002. GenBank accession numbers for DNA sequences derived from ex-type: ITS = MW732246; *tub2* = MW759031.

Etymology: Named after the host genus (*Pinus*) from which was isolated.

Distribution: Cantabria, Spain.

Known substrata: *Pinus radiata* needles.

Additional specimens examined: Puentenansa, Cantabria, Spain. (43°14′20.5"N 4°24′41.1"W), from symptomatic *Pinus radiata* needles, 21 November 2019, P. Monteiro, living culture CAA 1003.

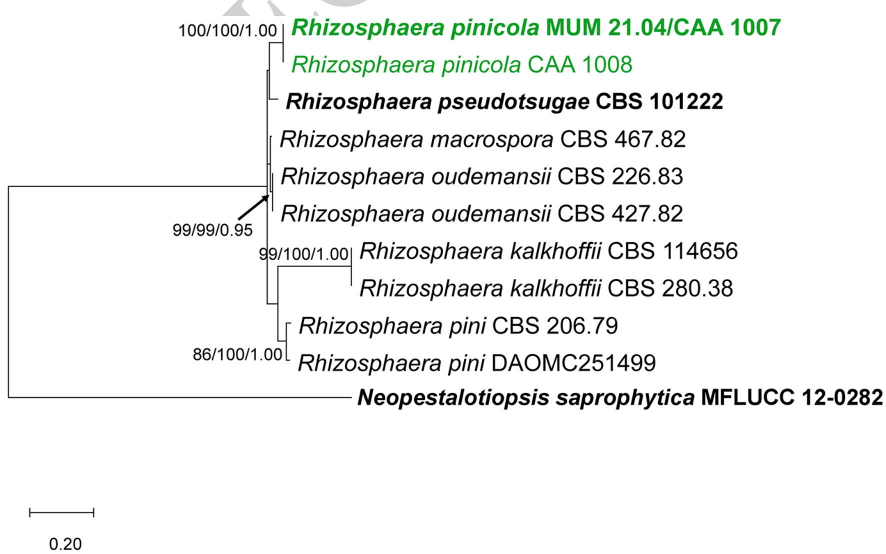

Fig. 6 Phylogenetic relationships of *Rhizosphaera* species based on combined ITS and tub2 sequence data and inferred using the maximum-likelihood (ML) method under the Kimura two-parameter model with gamma distribution. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Neopestalotiopsis saprophytica* MFLUCC 12-0282. Bootstrap values (≥ 70%) of ML and maximum-parsimony (MP) analyses and posterior probabilities (≥ 0.90) of Bayesian inference (BI) are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the new taxon proposed from the current study is given in green

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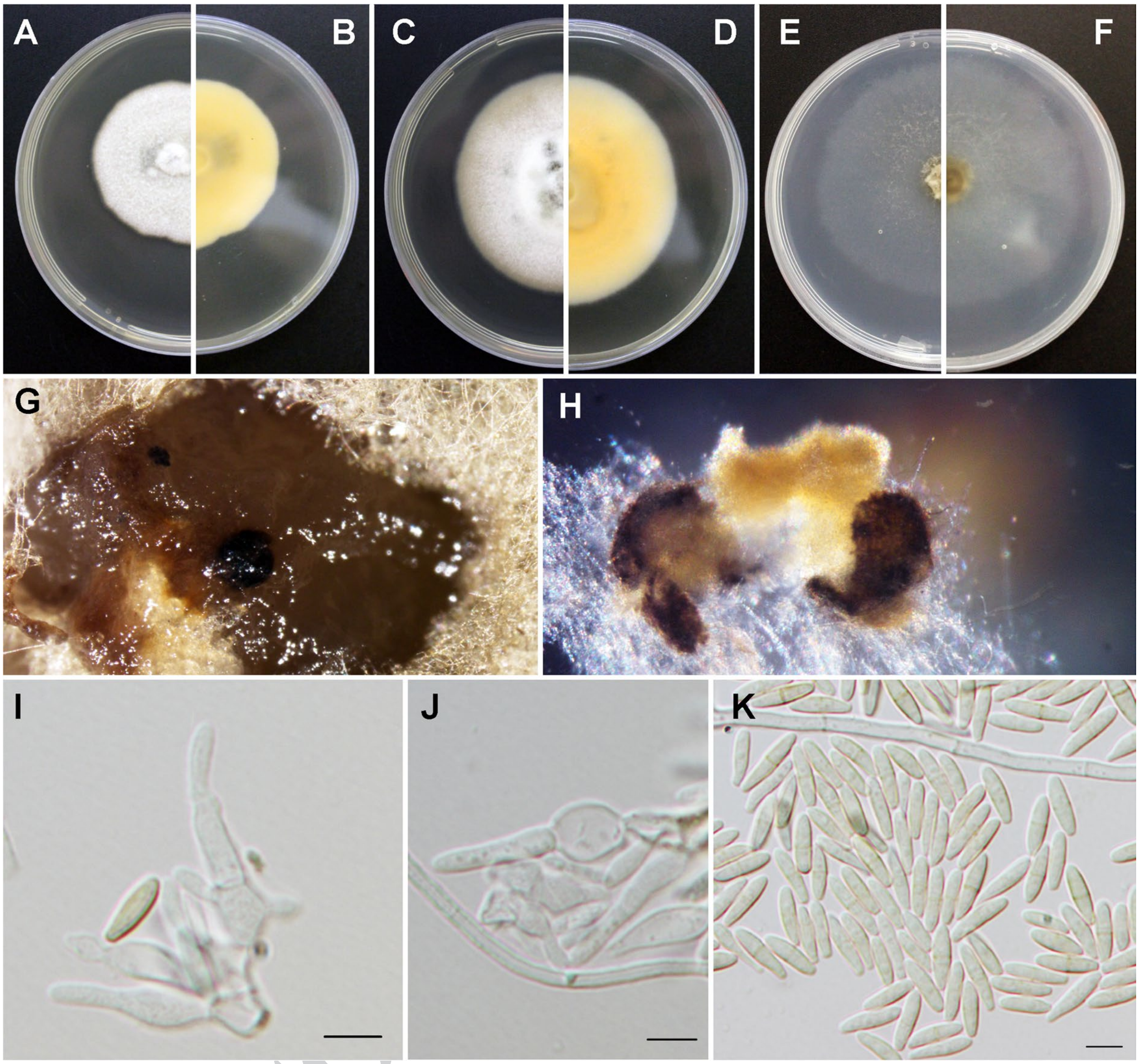

Fig. 7 *Didymocyrtis pini* (MUM 21.03). **A–B**. Colony after 14 days at 25 ºC on PDA (obverse and reverse). **C–D**. Colony after 14 days at 25 ºC on MEA (obverse and reverse). **E–F**. Colony after 14 days at 25 ºC on WA (obverse and reverse). **G**. Conidiomata on culture. **H**. Ruptured conidiomata with conidia mass. **I–J**. Conidiogenous cells with conidia. **K**. Conidia. Bars = 5 µm

GenBank accession numbers: ITS = MW732247; *tub2* = MW759030.

Description: Vegetative hyphae, 1.5 µm wide, thick-walled, septate, hyaline. Conidiomata submersed, brown to black, globose in WA. Conidiophores reduced to conidiogenous cells. Conidiogenous cells ampulliform, aseptate, hyaline, smooth walled, 7.3–9.0 × 3.1–5.7 µm (mean ± SD = 8.1 ± 0.9 × 4.0 ± 1.5 µm, n = 15). Conidia holoblastic, fusiform, smooth-walled, initially hyaline, guttulate and aseptate developing a central septum and then becoming olivaceous, 3.2–11.3 × 1.4–11.1 µm (mean ± SD = 8.5 ± 1.3 × 2.4 ± 1.0 µm, n = 100).

Culture characteristics: On 14 days old PDA, MEA and WA at 25 ºC, colonies growing regular with moderate aerial mycelium with 3.87 ± 0.10, 5.23 ± 0.18 and 5.95 ± 0.05 cm (diam), respectively. PDA and MEA obverse white with some tuffs olivaceous, periphery flesh red, reverse ochre yellow. WA obverse and reverse greyish white and greenish white to asparagus green in the plug area. At 35 ºC in MEA and WA, there was no visible growth.

Notes: Diederich et al. (2007) introduced the species *Phoma cladoniicola* (holotype: 20859A) (Diederich & Kocourkova, 2007). Later, Trakunyingcharoen et al. (2014) introduced a new genus, namely *Diederichomyces* to accommodate several species, including *P. cladoniicola* as *Diederichomyces cladoniicola* (CBS 128,023, CBS 128,025, CBS 128,026, CBS 128,027, CBS 131,731, CBS 131,732 and CBS 131,733) (Trakunyingcharoen et al., 2014). Ertz et al. (2015) resurrected the genus *Didymocyrtis* and synonymized it with the genera *Diederichia* and *Diederichomyces*. Therefore, a new combination was formed, namely *Didymocyrtis cladoniicola* (Ertz et al., 2015). Nonetheless, the connection to the type species of *D. cladoniicola* is unknown.

Didymocyrtis pini clustered in a distinct lineage in the genus *Didymocyrtis*, together with one isolate named as *D. cladoniicola* UTHSC:DI16-330 (Fig. 4). However, according to our phylogenetic analysis, isolates UTHSC:DI16-330 and UTHSC:DI16-313, which are identified as *D. cladoniicola* may have been wrong classified because they do not group with the well delimited clade of *D. cladoniicola*, that includes a series of cultures (CBS collection) that can be regarded as authentic and representative of *D. cladoniicola.* Furthermore, UTHSC:DI16-330 and UTHSC:DI16-313 were identified based only on D1-D2 of LSU sequences and according to these authors, none of these isolates grouped with *D. cladoniicola* CBS 128025 (Valenzuela-Lopez et al., 2017). In addition, there was no morphological data available associated with these two isolates to compare with *D. cladoniicola*.

Didymocyrtis pini is phylogenetically closely related to *D. branchylaenae* CPC 32,651 with high p-distances of nucleotide sites: 0.11 = 11% in ITS, and 0.09 = 9% in tub2 sequences. Micromorphologically, *D. pini* differs from *D. branchylaenae* in its conidia (8–)9–10(–13) × (2–)3 µm, color (medium brown for *D. branchylaenae* and olivaceous for *D. pini*) and septa (1–3 septa for *D. branchylaenae* and 0–1 for *D. pini*). Moreover, from the description of *D. cladoniicola* (CBS collection), *D. pini* differs substantially on conidiogenous cells (short-ampulliform, 2.5–4.5 × 2.5–4 µm for *D. cladoniicola* and 7.3–9.0 × 3.1–5.7 µm for *D. pini*) and conidia (ellipsoid, biguttulate, with a small guttule near each apex, (3.8–)4.7–5.9(–7.3) × (2.0–)2.4–3.0(–3.5) µm, l/b ratio (1.4–)1.7–2.2(–2.8) for *D. cladoniicola*).

Pestalotiopsis iberica P. Monteiro & M. Gonçalves, sp. nov. (Fig. 8)

MycoBank: MB 840199

Typification: Puentenansa, Cantabria, Spain. (43°12'27.6"N 4°25′08.2"W), isolated from symptomatic *Pinus radiata* needles, 21 November 2019, P. Monteiro, deposited in the Micoteca of Universidade do Minho (holotype a dried culture sporulating, MUM-H 21.02). Ex-type living culture MUM 21.02 = CAA 1004. GenBank accession numbers for DNA sequences derived from ex-type: ITS = MW732248; *tub2* = MW759035; *tef1-α* = MW759038.

Etymology: Named after the peninsula from which was isolated.

Distribution: Cantabria, Spain.

Known substrata: *Pinus radiata* and *P. sylvestris* needles.

Additional specimens examined: Puentenansa, Cantabria, Spain. (43°14′20.0"N 4°24′41.3"W), from symptomatic *Pinus sylvestris* needles, 21 November 2019, P. Monteiro, living culture CAA 1005. GenBank accession numbers: ITS = MW732250; *tub2* = MW759034; *tef1-α* = MW759037. Puentenansa, Cantabria, Spain. (43°12′29.2"N 4°25′05.7"W), from symptomatic *Pinus radiata* needles, 21 November 2019, P. Monteiro, living culture CAA 1006. GenBank accession numbers: ITS = MW732249; *tub2* = MW759036; *tef1-α* = MW759039.

Description: Hyphae 2 µm wide, smooth-walled, septate, hyaline. Conidiomata submersed or partially erumpent, globose, dark brown in PDA. Conidiophores reduced to conidiogenous cells, when present, septate and wider at the base, hyaline. Conidiogenous cells discrete or integrated, simple and erect from the base, hyaline, smooth walled, 13.1–23.3 × 1.6–3.4 µm (mean ± SD = 17.6 ± 2.9 × 2.4 ± 0.4 µm, n = 30). Conidia ellipsoid to fusoid, straight or slightly curved with 3- to 4-septa and occasionally slightly constricted, 16.1–30.7 × 4.5–7.3 µm (mean ± SD = 22.6 ± 3.1 × 5.7 ± 0.7 µm, n = 100). Apical cell hyaline, smooth-walled, conical to sub-cylindrical, 2.7–5.7 × 1.6–4.3 µm (mean ± SD = 4.2 ± 0.8 × 3.0 ± 0.6 µm, n = 100), with 2- to

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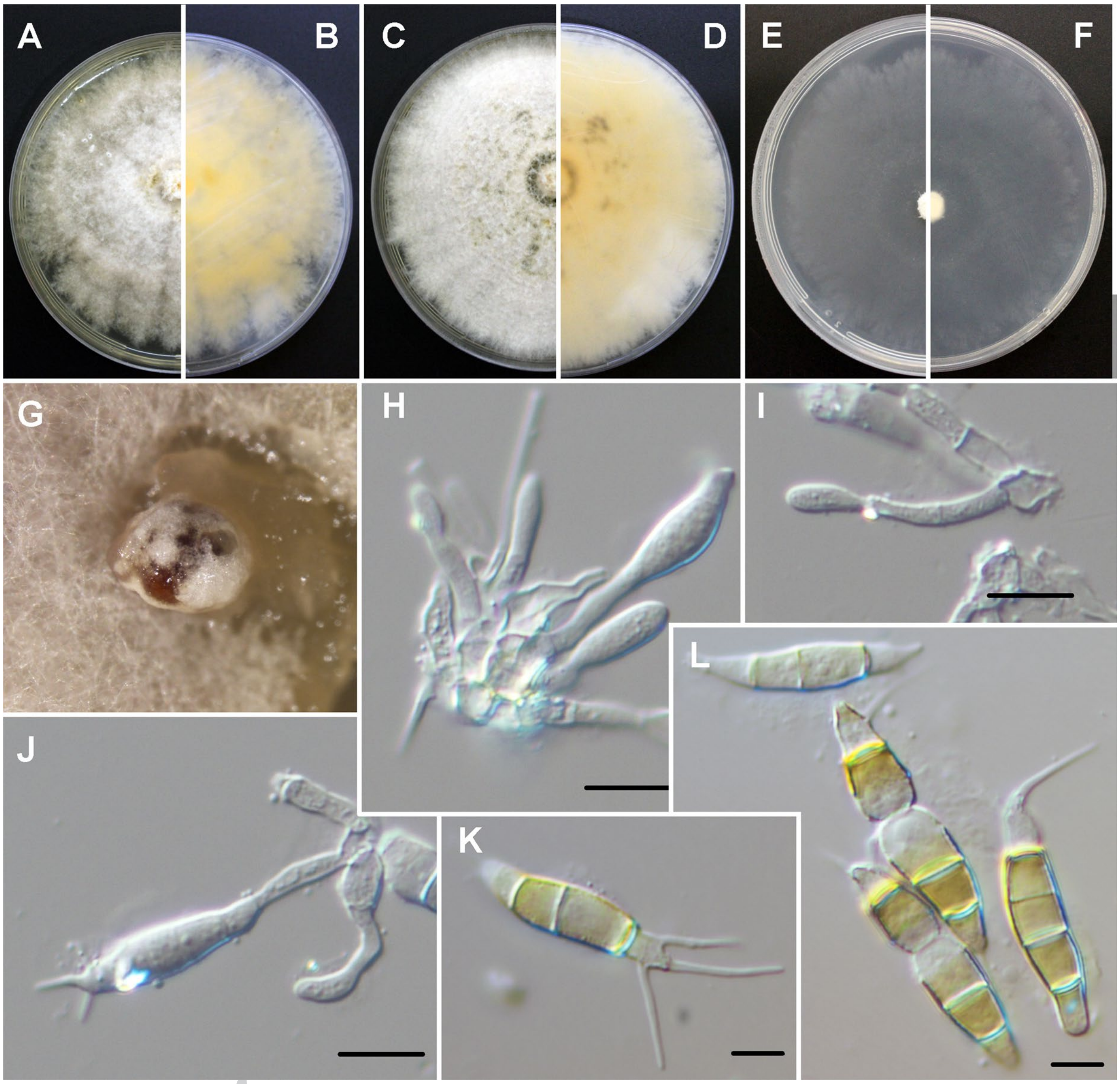

Fig. 8 *Pestalotiopsis iberica* (MUM 21.02). **A–B**. Colony after 14 days at 25 ºC on PDA (obverse and reverse). **C–D**. Colony after 14 days at 25 ºC on MEA (obverse and reverse). **E–F**. Colony after 14 days at 25 ºC on WA (obverse and reverse). **G**. Conidiomata on culture. **H–J**. Conidiophores. **K–L**. Conidia. Bars = H–J: 10 μm; K– L: 5 μm

3-tubular apical appendages (mostly 2), unbranched 3.8–16.0 μm (mean ± SD = 9.5 ± 2.4 μm, n = 100). Two to three median cells golden brown, smooth-walled, 9.8–19.3 × 4.2–3.8 μm (mean ± SD = 14.5 ± 2.4 × 5.6 ± 0.7 μm, n = 100). Basal cell hyaline, smooth-walled, 3.2–6.8 × 2.0–3.7 μm (mean ± SD = 4.6 ± 0.7 × 2.8 ± 0.4 μm, n = 100), with a single basal appendage filiform 1.4–10.2 μm (mean ± SD = 4.5 ± 1.8 μm, n = 100).

Culture characteristics: On 14 days old PDA, MEA and WA at 25 ºC, colonies growing regular with cottony aerial mycelium with 8.5 ± 0.00, 8.5 ± 0.00 and 5.85 ± 2.43 cm (diam), respectively. PDA and MEA obverse whitish to pale salmon, reverse pale salmon to salmon. WA obverse and reverse greyish white. At 35 ºC in PDA, MEA and WA and 5 ºC in PDA and WA, there was no visible growth.

Notes: *Pestalotiopsis iberica* is phylogenetically closely related to *P. lushanensis* LC 4344 and LC 8182 with 16, 5 and 0 nucleotide differences in ITS, *tub2* and *tef1-α* sequences, respectively. Micromorphologically *P. iberica* differs from *P. lushanensis* in its width conidia (22.3 ± 1.9 × 8.6 ± 0.6 for *P. lushanensis*), color of median cells (pale brown to brown for *P. lushanensis* and golden brown for *P. iberica*), apical appendages' length (20.3 ± 2.9 µm for *P. lushanensis* and 9.5 ± 2.4 µm for *P. iberica*) and number of tubular apical appendages (mostly 3 for *P. lushanensis* and mostly 2 for *P. iberica*.

Rhizosphaera pinicola P. Monteiro & M. Gonçalves, sp. nov. (Fig. 9)

MycoBank: MB 840200

Typification: Puentenansa, Cantabria, Spain. (43°12′27.4"N 4°25′09.0"W), isolated from symptomatic *Pinus nigra* needles, 21 November 2019, P. Monteiro, deposited in the Micoteca of Universidade do Minho (holotype a dried culture sporulating, MUM-H 21.04). Ex-type living culture MUM 21.04 = CAA 1007. GenBank accession numbers for DNA sequences derived from ex-type: ITS = MW732245; *tub2* = MW759033.

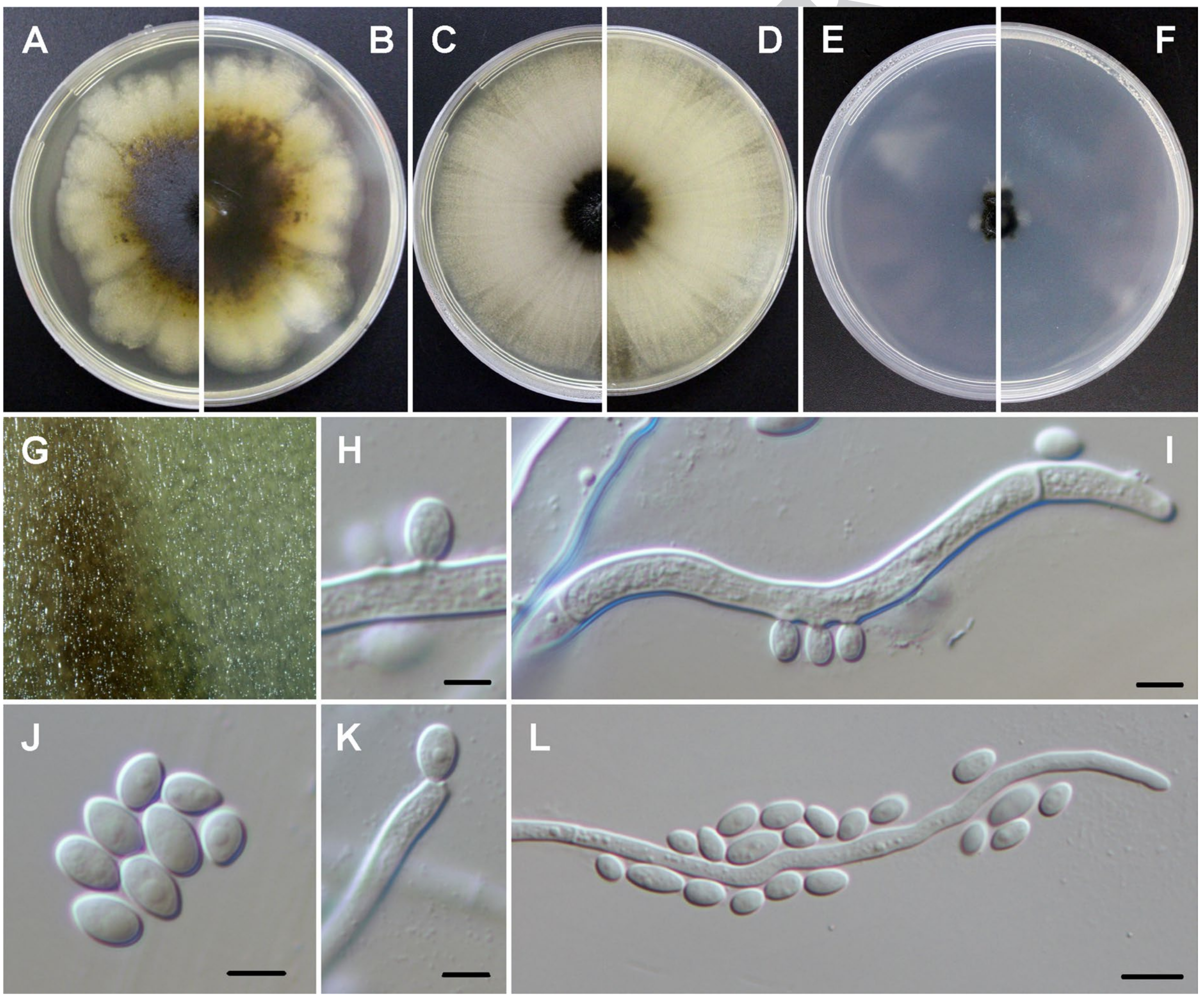

Fig. 9 *Rhizosphaera pinicola* (MUM 21.04). **A–B**. Colony after 14 days at 25 ºC on PDA (obverse and reverse). **C–D**. Colony after 14 days at 25 ºC on MEA (obverse and reverse). **E–F**. Colony after 14 days at 25 ºC on WA (obverse and reverse). **G**. Colony texture on PDA. **H–I, K**. Conidia borne directly on the wall of hyphae. **J–L**. Conidia. Bars = H–K: 5 µm; L: 10 µm

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Etymology: Named after the host genus (*Pinus*) from which was isolated.

Distribution: Cantabria, Spain.

Known substrata: *Pinus nigra* and *P. radiata*.

Additional specimens examined: Puentenansa, Cantabria, Spain. (43°14′20.8"N 4°24′40.3"W), from symptomatic *Pinus radiata* needles, 21 November 2019, P. Monteiro, living culture CAA 1008. GenBank accession numbers: ITS = MW732245; *tub2* = MW759032.

Description: Hyphae 5 µm wide, thick-walled, septate, hyaline. Conidiophores and conidiogenous cells absent in PDA. Conidia borne directly on the wall of hyphae, aseptate, smooth, ovoid to oval, vacuolate, hyaline, 4.4–8.6 × 2.9–5.2 µm (mean ± SD = 6.0 ± 0.7 × 3.8 ± 0.4 µm, n = 100).

Culture characteristics: On 14 days old PDA, MEA and WA at 25 ºC, colonies growing regular with cottony aerial mycelium with 6.58 ± 0.33, 8.5 ± 0.00 and 1.32 ± 0.49 cm (diam), respectively. PDA obverse and reverse blackish brown on the center, yellowish white at the edge with a smooth wax yellow transition. MEA obverse and reverse yellowish white, with blackish brown on the center (with smaller area when compared to PDA). WA obverse and reverse blackish brown. At 35 ºC in PDA, MEA and WA and 5 ºC in WA, there was no visible growth.

Notes: *Rhizosphaera pinicola* is phylogenetically closely related to *R. pseudotsugae* CBS 101222 with high p-distances of nucleotide sites: 0.01 = 1% in ITS, and 0.111 = 11.1% in *tub2* sequences. Micromorphologically, *R. pinicola* differs from *R. pseudotsugae* in its conidia size: bigger [(7–)8–11(–12) × (3.5–)4–5.5(–6) µm] than *R. pinicola*.

Discussion

Plants harbour a wide range of microorganisms establishing mutualistic relationships between them that might result in either beneficial or harmful outcomes (Trivedi et al., 2020). In this study, several fungal species have been isolated and identified from symptomatic pine needles presenting spots/bands with visible fruiting bodies. The same symptomatology has been associated with different needle diseases affecting several conifer species, such as Dothistroma Needle Blight, Brown-spot needle blight, Rhizosphaera needle cast, Lophodermium needle cast or Cyclaneusma needle cast (Bednářová et al., 2013).

One of the new species described in this study, *Didymocyrtis pini*, belongs to the genus *Didymorcyrtis* that has undergone several taxonomic rearrangements in recent years (Ertz et al., 2015). Initially, Diederich and Kocourkova (2007) introduced the *Phoma cladoniicola* sp. nov., which was later accommodated in a newly formed genus, named *Diederichomyces* (Trakunyingcharoen et al., 2014). In 2015, Ertz et al. resurrected the genus *Didymocyrtis* and synonymized it with genera *Diederichia* and *Diederichomyces* (Ertz et al., 2015). The genus *Didymocyrtis* is considered to be a lichenicolous fungus (Ertz et al., 2015), meaning that these fungi colonize lichens in nature, forming obligate relationships that goes from saprotrophs (colonizing death tissues) to parasites (colonizing living tissues to obtain carbon sources) (Lawrey & Diederich, 2003; Lawrey et al., 2012). Parasitic associations may range from nonaggressive to more severe virulent types leading to lesions or discolorations in hosts' tissues (Lawrey & Diederich, 2003). All species included in *Didymocyrtis* genus have been identified as lichenicolous fungi (Diederich et al., 2018). The only exception is *D. brachylaenae* that was isolated from *Brachylaena discolor*, which is a plant species from the family *Asteraceae* (Crous et al., 2018). Although *Didymocyrtis* species are usually found associated with lichens, the family it belongs (*Phaeosphaeriaceae*) includes several economically important plant pathogens (Phookamsak et al., 2014). Therefore, it is not surprising the association of *D. pini* with plant tissues (*Pinus radiata* needles). To our knowledge this is the first time that a *Didymorcyrtis* species was isolated from *Pinus* species.

Pestalotiopsis is a fungal genus known as a plant pathogen and responsible for causing a wide range of symptoms in their hosts ranging from leaf blights/chlorosis, tip and/or shoot diebacks and cankers, being appointed as responsible of large economic losses (Maharachchikumbura et al., 2014). Despite being considered a plant pathogen, members of this genus also manage to live within plant tissues as endophytes or occur as saprobes (living of death plant tissues) (Maharachchikumbura et al., 2014). *Pestalotiopsis* spp. has been found in a wide range of plants hosts and environments (Maharachchikumbura et al., 2014). Regarding forest species, *Pestalotiopsis*

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spp. has been found in *Eucalyptus* spp. (Morales-Rodríguez et al., 2019), *Cupressus* spp. (Madar et al., 1991) and *Pinus* spp. (Ivanová, 2016; Magnani et al., 2003; Qi et al., 2021; Silva et al., 2020; Zamora et al., 2008). Therefore, it is not uncommon that in this study a species of *Pestalotiopsis* was successfully isolated from needles of *Pinus radiata* and *P. sylvestris*. *Pestalotiopsis* sp. were previously successfully isolated from *P. radiata* plant tissues (xylem and phloem) and *Pestalotiopsis neglecta* from needles and twigs of *P. sylvestris* var. *mongolica* (Bezos et al., 2018; Jie et al., 2020). *Pestalotiopsis bessey* was also sucessfully isolated from *P. halenpesis* (Botella & Diez, 2011), while *Pestalotiopsis funerea* was isolated from *P. pinaster* needles (Martínez-Álvarez et al., 2012).

In the present study, we also observed *Pestalotiopsis pini* infecting *P. sylvestris* and *P. radiata*, and *Pestalotiopsis verruculosa* in *P. pinaster* (Fig. 3). *Pestalotiopsis pini* was found in association with *P. pinea* and *P. pinaster* needles in Portugal showing shoot blight, trunk necrosis, needle blight and pine cone decay (Silva et al., 2020). Other authors reported this species also in *Pinus* spp. in USA and Chile (Liu et al., 2019). *Pestalotiopsis verruculosa* was isolated for the first time from dead plant tissues from different sites in China (Maharachchikumbura et al., 2012). Despite its relevance as a plant pathogen, the genus *Pestalotiopsis* has been gaining interest from the scientific community as a producer of interesting secondary bioactive compounds. Several species of *Pestalotiopsis* are able to produce distinct bioactive compounds (e.g. antioxidants, immunosuppressants, anticancer agents) with potential interest as future drug formulations (for human or plant applications) (Xu et al., 2010).

Rhizosphaera needle cast (RNC) is a fungal disease primary caused by *R. kalkhoffii* Bubák (1914), being already reported in both natural and planted stands, in *Picea* spp., *Pinus ponderosa, Pinus thunbergii* and *Pseudotsuga menziesii* (Goldberg, 2017; Juzwik et al., 1993; Kumi & Lang, 1979; Skilling & Walla, 1986). It was first identified in 1938 in Connecticut, USA in ornamental blue spruce (*Picea pungens*) being later found in almost all country (Skilling & Walla, 1986). Trees infections usually takes place during spring and symptoms usually become visible in the spring/summer of the following year and are promoted by spores carried out by rain splashes from infected needles (Skilling & Walla, 1986). Typical symptoms of this disease include needle discoloration that in summer turn start to turn yellowish, becoming brownish eventually falling-off the tree (giving name to the disease) and leading to branch defoliation and ultimately leading to trees death (Skilling & Walla, 1986). Typically, RNC affects trees' oldest needles, mainly in the lower portion of the canopy progressing upwards and it is capable of infect trees of all ages (Skilling & Walla, 1986). Other *Rhizosphaera* species have been associated with this needle symptomatology. In 1981, *R. oudemansii* was successfully isolated from Spanish fir, *Abies pinsapo* Boiss, study area in Sierra del Endrinal (Cadiz, Spain) from diseased needle stomata (Martínez & Ramírez, 1983). In a survey carried out in Delaware, USA, *R. pini* was found in *P. thunbergi* infected with *Bursaphelenchus xylophilus* (pine wilt nematode) (Adams & Orehart, 1982).

Other fungal species found in this work have already been isolated from pine needles in previous studies. *Epiccocum nigrum* and *Sydowia polyspora* were successfully isolated from *P. sylvestris* and *P. radiata* samples to be tested as antagonists against *Fusarium circinatum* (Martínez-Álvarez et al., 2016). *Sydowia polyspora* was also successfully isolated from *P. radiata* (with symptoms of pine pitch canker disease), *P. nigra* and *P. sylvestris* (without pine pitch canker disease symptomatology) (Muñoz-Adalia et al., 2017). In another study, the endophytic mycobiota of *P. sylvestris* was explored and isolated 143 fungal taxa among which were included *E. nigrum*, *Cladosporium cladosporioides*, *Cladosporium herbarum* and *Umbelopsis isabellina* (Giordano et al., 2009). *Epicoccum nigrum* and *C. herbarum* were also isolated from *P. sylvestris* and *P. pinaster* needles (Martínez-Álvarez et al., 2012). In another study, *P. sylvestris* needles mycobiota with symptoms of the autumn needle cast was screened, and *E. nigrum* and *C. cladosporioides* were present (Kowalski, 1993). Other two species, such as *Cladosporium colombiae* and *Cladosporium perangustum* were also reported associated to plants, including *Cortaderia, Eucalyptus* sp., *Acacia* sp. and *Musa* sp. (Bensch et al., 2010; Schubert et al., 2009). We also observed *C. colombiae* and *C. perangustum* associated to *P. radiata* and *P. pinaster*. In this study, an isolate belonging to *Fusarium* genus was also successfully retrieved from the field samples which goes into accordance to the fact that *Fusarium* spp. are well recognized as plant

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associated fungi (Summerell, 2019). Other species found in this study associated to *P. radiata* was *Neophysalospora eucalypti,* which was also reported in leaves of *Corymbia henryi* (*Myrtaceae*) and cutting rot of *Eucalyptus grandis*×*camaldulensis* (Crous et al., 2014); *Botrytis cinerea*, a well-known plant facultative parasite, being already found in several *Pinus* spp. including *P. radiata* and *P. sylvestris* (Bußkamp et al., 2020; Mercader et al., 2006; Mittalet al., 1987); and *Pseudoalternaria rosae,* previously identified as *Alternaria rosae* (Lawrence et al., 2014). Lastly, we also observed *Penicillium corylophilum* in *P. pinaster* and to the best of our knowledge this is the first time that *Penicillium corylophilum* is identified in *P. pinaster*.

Conclusions

The study carried out allowed to identify the fungal diversity associated, together with the description of three new fungal species—*Didymocyrtis pini* sp. nov., *Pestalotiopsis iberica* sp. nov. and *Rhizosphaera pinicola* sp. nov., with a specific set of symptoms (spots or bands with fruiting bodies). From the studies above-mentioned, *Rhizosphaera* spp. and *Pestalotiopsis* spp. could have an impact on several coniferous, threatening their healthiness and productivity among both natural and planted stand and nurseries. However, further studies are required to clarify the level of pathogenicity of this new species (including *Didymocyrtis pini*) to *Pinus* spp., as well as other coniferous, and to understand the mechanisms behind these interactions in order to adopt eco-friendly management measures and methodologies, thus preventing future potential outbreaks.

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All principles of ethical and professional conduct have been followed during this research and elaboration of this manuscript.

Declarations

Research involving human participants and/or animals Not applicable.

Informed consent All authors have reviewed the manuscript and approved its submission to the European Journal of Plant Pathology.

Conflict of interest The authors declare that they have no conflict of interest.

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